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Compatibility of released and adventive populations of *Ganaspis kimorum* Buffington, 2024, (Cynipoidea, Figitidae), parasitoid of the spotted-wing drosophila *Drosophila suzukii* (Matsumura, 1931)

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Abstract

Taxonomic and host associations have been closely studied within the *Ganaspis brasiliensis* (Ihering, 1905) (Hymenoptera, Figitidae) complex as parasitoids of the spotted-wing drosophila, *Drosophila suzukii* (Matsumura, 1931) (Diptera, Drosophilidae). Initially, five genetic groups (G1–G5) were identified that suggested the existence of cryptic species that vary in their host ranges and geographic distributions. What was referred to as the "G1" strain was recently described as *G. kimorum* Buffington, 2024, and approved for release as a classical biological control agent in the United States and parts of Europe. Concurrently, an adventive population of *G. kimorum* was found in British Columbia, Canada and is likely spreading through parts of the Pacific Northwest such as Washington State, USA. Here, we compare the reproductive compatibility and molecular similarity of laboratory-bred *G. kimorum* (collected in Tokyo, Japan) used for release in the USA and Europe with the adventive population found in Washington State, USA. Cross-breeding experiments between the Tokyo and the adventive population showed successful mating and the production of female offspring, indicating that they are reproductively compatible. For both populations, the mitochondrial *COI*

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barcode region was sequenced and further confirmed the conspecificity of the Tokyo and adventive Washington populations with published *G. kimorum*. These findings will help to better understand and document the effects of releases of *G. kimorum* and the reproductive success of adventive and released populations.

Keywords
Biological control, cryptic species, reproductive compatibility

Introduction

Some insect species are widely distributed geographically and, as a result, isolated populations can exhibit biological, ecological, and genetic differences due to selective environmental pressure and local adaptation (Hopper et al. 1993). Such intraspecific variability can lead to the differentiation of populations characterized by varying levels of reproductive compatibility (Hopper et al. 1993; Turelli et al. 2001), as well as different biological characteristics that could, for example, impact their effectiveness as biological control agents (Stouthamer et al. 2000; Vallina et al. 2020). Moreover, mating incompatibility among parasitoid biotypes is often attributed to the presence of cryptic species that exhibit differences in genetic, physiological, behavioral, and/or ecological traits, despite morphological similarity (Bickford et al. 2007; Heraty et al. 2007). For example, there is an increasing realization that maternally inherited endosymbionts, such as *Wolbachia* or *Cardinium* present in arthropods, can cause cytoplasmic incompatibility (e.g., Perlman et al. 2014; Gebiola et al. 2016; Bruzzese et al. 2022). In such cases, cross-breedings between males infected with the endosymbiont and uninfected females or females infected with a different endosymbiont strain result in reproductive incompatibility.

In this context, understanding the reproductive status among populations of biological control agents can be crucial to predict the success of multiple introductions and subsequent admixture impact on pest control efforts (Stouthamer et al. 2000; Dayrat 2005; Gebiola et al. 2016). To this aim, laboratory cross-breeding experiments and analyses of genetic diversity of different geographical populations through DNA barcode sequencing or genome assembly can play an essential role in the taxonomic identification and detection of cryptic species, especially for parasitoids employed as biological control agents (Desneux et al. 2009; Seehausen et al. 2020). Here, we investigate two populations of *Ganaspis kimorum* Buffington, 2024 (Hymenoptera, Figitidae), one of the main parasitoids attacking the spotted-wing drosophila, *Drosophila suzukii* (Matsumura, 1931) (Diptera, Drosophilidae). When it was first reported from Japanese field collections of wild cherries infested by *D. suzukii*, it was assigned as the *suzukii*- type of *G. xanthopoda* Ashmead (Kasuya et al. 2013) and then later as *G. brasiliensis* (e.g., Buffington and Forshage 2016; Daane et al. 2016; Giorgini et al. 2019) or *G.* cf. *brasiliensis* (e.g., Girod et al. 2018a; Seehausen et al. 2020). Nomano et al. (2017) divided the *G.* cf. *brasiliensis* complex, based on DNA sequences of the *COI* barcode region, into five groups (G1–G5) and reported that two *G.* cf. *brasiliensis*

groups (G1 and G3) were reared from *D. suzukii* in Asia. Later surveys confirmed that G1 and G3 successfully attacked *D. suzukii* and often co-occur at the same collection sites (Giorgini et al. 2019; Daane et al. 2021), but based on laboratory test have different host ranges with G1 being more specialized on *D. suzukii* than G3 (Girod et al. 2018b; Daane et al. 2021). Based on cross-breeding experiments showing G1 and G3 mating incompatibility, detailed molecular work (Gariepy et al. 2024; Hopper et al. 2024; Seehausen et al. 2024), and taxonomic differences in the ovipositor, G1 and G3 were determined to be separate species and named *G. kimorum* and *G. lupini*, respectively (Sosa-Calvo et al. 2024).

As the most host specific Asian *D. suzukii* parasitoid, *G. kimorum* was selected for release as a biological control agent in parts of Europe (Fellin et al. 2023) and the USA (Gariepy et al. 2024), with field release approved for *G. kimorum* (initially as *G. brasiliensis* G1 (Tokyo)) and began in Italy in 2021 (Lisi et al. 2022) and the USA in 2022 (Gariepy et al. 2024). Concurrently, an adventive population of *Ganaspis* sp. (initially reported as *G.* cf. *brasiliensis* G1) was discovered in Northwestern North America, first in British Columbia, Canada (Abram et al. 2020), and later across the border in Washington, USA (Beers et al. 2022) and is likely to expand to other regions in the USA. All specimens of the adventive *Ganaspis* sp. attacking *D. suzukii* in the Pacific Northwest have thus far been identified (using morphology and DNA barcoding) as *G. kimorum*, still it is important to confirm if the adventive population is similar to *G. kimorum* collected in Japan and being released across the USA. Moreover, given the wide distribution of *G. kimorum* in East Asia (Daane et al. 2016; Girod et al. 2018b; Giorgini et al. 2019), understanding its genetic diversity and potential admixture impact is crucial at the early stage of this biological control program for *D. suzukii*. Here, we aimed to confirm the reproductive compatibility of the released and adventive populations of *G. kimorum* and confirm their molecular similarity or dissimilarity through barcoding. This will provide baseline information for the status of the *G. kimorum* as it pertains to predictions for biological control success.

Material and methods

Rearing of *Ganaspis kimorum*

Two colonies of *G. kimorum* were maintained at the University of California Kearney Agricultural Research and Extension Center in California. The first colony was established from material originally collected in Tokyo, Japan, in 2015 (Girod et al. 2018b; released in the USA and Italy as a biological control agent: 'rel'), the second was collected in Washington, USA, in 2020 (Beers et al. 2022; adventive North American population: 'adv'). Both colonies were maintained at 25 ± 5 °C, 35 ± 5% relative humidity and 16:8 L:D photoperiod and rearing protocols were similar. Following Rossi-Stacconi et al. (2022), 10–15 fresh, rinsed blueberries were placed on paper towels in

plastic containers (80 mm high, 90.5 mm diameter) with mesh lids and exposed to around 50 *D. suzukii* (50:50 sex ratio) for 24–72 h. Cotton wicks soaked in sugar water served as nutrition for the adult flies. Then, the eggs and the hatched *D. suzukii* larvae were exposed to the parasitoids by adding around 12 *G. kimorum* (50:50 sex ratio) to each container for 72 h. Cotton wicks soaked in honey water and honey droplets on the mesh lid replaced every second day served as nutrition for the adult parasitoids. After the parasitoids were removed, the paper towels were kept moist until *G. kimorum* offspring emerged and adult *G. kimorum* were then collected daily by aspiration and immediately used for the colony. The rearing containers described above were placed in mesh cages ('BugDorm-4090 Insect Rearing Cage 47.5 × 47.5 × 47.5 cm', MegaView Science Co. Ltd., Taichung, Taiwan) as an additional barrier to separate the two colonies. All cages were inspected before opening and if there were any stray wasps that had escaped a container they were removed. We had separate handling cages for transferring *G. kimorum* individuals from the two colonies to prevent escapees and contamination and transferred them with mouth aspirators only used for the specific colony. Whenever possible, one person worked on one colony, and afterwards a second person on the other.

Cross-breeding experiment

The two *G. kimorum* populations were cross-bred in 2022–2023 with the following treatments: 'rel' female × 'adv' male, and 'adv' female × 'rel' male. To establish what proportion of *G. kimorum* females produces female offspring under the experimental conditions and also possible cytoplasmic incompatibility, the controls 'rel' female × 'rel' male, and 'adv' female × 'adv' male were added. If cytoplasmic incompatibility occurs, cross-breedings between infected males with uninfected females can result in reproductive incompatibility. Unmated individuals of *G. kimorum* were collected the day they emerged and placed with a mate for 1 h inside a plastic vial (70 mm high, 20.5 mm diameter) with a sponge lid that was coated in a thin layer of honey. They were observed for the first 10 min, and once every 10 min after that within the hour. The occurrence of mating behavior was recorded when mating was observed. After 1 h, the couple was placed into a rearing container, as previously described, that contained blueberries with 0–24 h old *D. suzukii* eggs and larvae. After 72 h, the parasitoids were removed and placed into 1.5 ml Eppendorf tubes filled with 90% ethanol to be stored at -80 °C. Emerged offspring was sexed and counted. Since *G. kimorum* is haplodiploid, meaning that males emerge from unfertilized eggs and females from fertilized eggs, the presence of daughters indicates successful mating. To confirm the viability of emerged daughters, replicates with both female and male offspring were used for a secondary experiment. Offspring were collected and stored in vials with honey until females and males had had at least 24 h of time to mate before one female and one male from each vial were placed onto new blueberries with *D. suzukii* immatures, as described previously. Only a maximum of three daughters per replicate was used to establish F1 viability. Afterwards, all F1 offspring were similarly stored in 90% ethanol.

Statistical analyses of cross-breeding experiment

The proportion of female *G. kimorum* producing daughters was compared between treatments and controls with a generalized linear model (GLM) with a binomial error distribution. Only cross-breedings that produced offspring were included. The relationship between observed mating and the occurrence of female offspring was investigated with a Pearson's product-moment correlation. All statistics were conducted in R version 3.6.2 (R Core Team 2020) and RStudio version 1.2.5033 (RStudio Team 2016) as well as package 'car' (Fox et al. 2016).

Barcoding

A total of 127 adult parasitoids were used for DNA barcoding. DNA was extracted from the whole body of each specimen using the FastDNA® kit and the FastPrep® Instrument (MP Biomedicals, Santa Ana, California, USA), according to the manufacturer's instructions, with minor modifications. Extracted DNA was quantified using a Nanodrop 2000c spectrophotometer (Thermo Fischer Scientific, USA), diluted to approximately 50 ng/μL and stored at -20 °C.

The barcode region of the mitochondrial coding gene Cytochrome Oxidase subunit 1 (*COI*) was amplified with the universal primer pairs LCO/HCO (LCO: 5'- GGTCAACAAATCATAAAGATATTGG – 3' and HCO: 5' – TAAACTTCAGGGTGACCAAAAAATCA - 3', 440–688 bp) (Folmer et al. 1994). Polymerase chain reaction (PCR) was conducted in a 20 μl mixture containing 10 μl of GoTaq® Green Master Mix (Promega, Madison, Wisconsin, USA), 2 μl of extracted DNA, 0.5 μl of each primer and ultrapure water up to the final volume.

The *COI* target region was amplified by setting the following profile of the T100 Thermal Cycler (Bio-Rad): 94 °C for 10 min, 35 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1.5 min, followed by final extension at 72 °C for 1.5 min. PCR products were sent to the University of California, Berkeley DNA sequencing facility for direct sequencing of both strands using the ABI Big Dye V3.1 terminator sequencing reaction kit (Perkin-Elmer/ABI, Weiterstadt, Germany) on an ABI 3707xl DNA Analyzer (Perkin-Elmer) with POP 7 and a 50 cm array. A Basic Local Alignment Search Tool (BLAST) comparison of amplified *COI* sequences was performed using the NCBI database.

Sequences were trimmed using FinchTV (Geospiza, Inc., Seattle, Washington, USA) software by removing terminal ambiguous regions and a multiple sequence alignment was performed using the MUSCLE algorithm (Edgar 2004) in MEGA (version 11) (Tamura et al. 2021). Forty-two available *COI* sequences of *G.* cf. *brasiliensis* were downloaded from the NCBI database in March 2023, including those originating from Nomano et al. (2017), Giorgini et al. (2019) and Abram et al. (2020), and then added to the dataset before analysis.

A neighbor-joining tree (Saitou and Nei 1987) was constructed using the Maximum Composite Likelihood method with 1000 bootstrap replicates in MEGA (version 11) (Tamura et al. 2021). A *G. xanthopoda* (Ashmead, 1896) sequence was included in the analysis as the outgroup.

Results

Cross-breeding experiment

All four cross-breedings yielded *G. kimorum* offspring (Table 1). The production of daughters suggests that successful mating had occurred. There were no significant differences between the cross-breedings in the proportion of females that produced daughters (GLM, DF = 3, 55, $\chi^2 = 0.74$, $p = 0.864$).

Sixty-three percent of the females producing offspring were observed to have mated with their male during the first hour. There was no correlation between observed mating and the occurrence of female offspring (Pearson's correlation, t = -0.41, DF = 57, $p = 0.684$); some females that had not been observed mating produced daughters and vice versa.

The majority of the F1 generation did not produce offspring. The one daughter from the 'rel' female × 'adv' male cross-breeding that did produce offspring, also produced female offspring. Similarly, the two cross-breedings between 'adv' females x 'rel' males F1 generation producing offspring included daughters, indicating viable original cross-breedingss.

Barcoding

The molecular characterization based on the mitochondrial *COI* gene confirmed that generated high quality reads from specimens showed 99–100% sequence similarity with *G. brasiliensis* isolate USNMENTO (accession number: MT559420.1), *G. xanthopoda* isolate suz21 (accession number: LC122451.1) and isolate suzukii type 3 (accession number: AB678736.1), and sequences were submitted to GenBank (accession number: PP980973 and PQ493166–PQ493291). Moreover, both the parental 'rel' and 'adv' *G. kimorum* used in the cross-breeding experiment matched with the same accession numbers showing a high sequence similarity (99–100%).

The phylogenetic tree showed two main clusters with an extended group referring to the *G. kimorum* samples (Fig. 1, blue). The *G. kimorum* cluster included the cross-breedings of the 'rel' and 'adv' *G. kimorum* in this work, as well as samples of *G.* cf. *brasiliensis* collected both in Japan and China and reported in Nomano et al. (2017) and Giorgini at al. (2019). The second extended cluster showed a substructure consisting of a clade made of the *G.* cf. *brasiliensis* G2 sequences (LC122441.1 and LC122440.1; Fig. 1, red) and another clade incorporating sequences of *G. lupini*, *G.* cf. *brasiliensis* G4, and *G. brasiliensis* s. str. (Fig. 1, yellow, orange, green).

Table 1. Females producing daughters after cross-breedings. 'rel' = released population, 'adv' = adventive population, 'WA' = Washington State, USA.

Cross-breeding	Origin of female	Origin of male	Parental females (n)	Total progeny (n)	Females producing offspring (n)	Females producing daughters (n)	Females producing daughters (%)
'rel' × 'adv'	Tokyo	WA	68	143	25	18	72.0
'rel' × 'adv'	WA	Tokyo	28	29	9	7	77.8
'rel' × 'rel'	Tokyo	Tokyo	36	125	17	11	64.7
'adv' × 'adv'	WA	WA	22	29	8	5	62.5

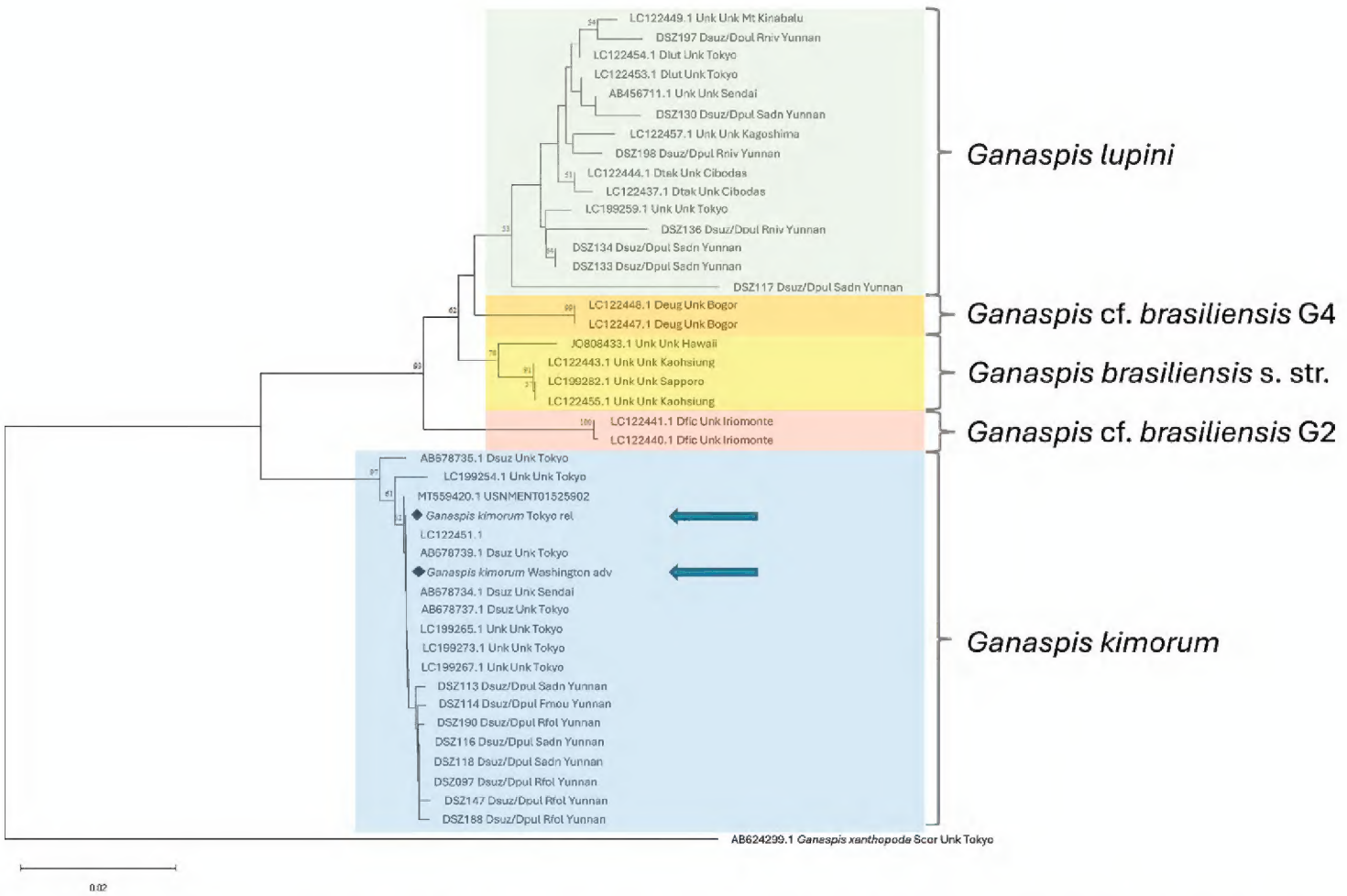

Figure 1. Neighbor-joining tree; Neighbor-joining tree based on *COI* sequences for *G. kimorum* specimens used in the cross-breeding experiment (arrows) and on sequences retrieved from NCBI. The accession number, collection locality and host are also reported for each sequence. *Ganaspis xanthopoda* AB624299 was used as outgroup. Bootstrap values are indicated on the branches (values < 50% are not shown).

Discussion

Our results confirm that the *G. kimorum* currently being released in the framework of the classical biological control program against *D. suzukii* is reproductively compatible with the adventive population of *G. kimorum* reported in North America. This was confirmed by cross-breeding of the adventive and the imported *G. kimorum* populations producing female progeny providing evidence for reproductive compatibility, and with the clustering of the *COI* barcoding region.

Taxonomic status and reproductive compatibility of introduced parasitoids and previously released, adventive or native parasitoids can be crucial for the outcome of biological control programs (Vallina et al. 2020). For example, the presence of incompatible populations of biological control agents inhabiting the same ecological niche can lead to competition (Paterson et al. 2016). On the other hand, admixture between different populations can introduce new genetic variations and potentially enhance their adaptability to diverse environments (Chapple et al. 2013; Dlugosch et al. 2015). The majority of the F1 generation in our study did not produce offspring. Since this occurred in both controls, we attribute this to the rearing conditions but with the cross-bred F1 generations producing some offspring a change through admixture could be at play. However, the benefits of admixture are not guaranteed and can

be limited by factors such as genetic divergence and the timing of admixture (Rincon et al. 2006; Havill et al. 2012). For example, while admixture can introduce beneficial alleles, it can also lead to outbreeding depression or the dilution of local adaptations (Barker et al. 2018). If the introduced population has low genetic diversity to begin with, admixture may not provide significant benefits as there are fewer beneficial alleles to mix (Dlugosch et al. 2015; Barker et al. 2018).

Given the wide distribution of *G. kimorum* in East Asia (Daane et al. 2016; Girod et al. 2018b; Giorgini et al. 2019), understanding its genetic diversity and reproductive compatibilities among geographical populations is crucial for effective biological control of *D. suzukii*. The Asian origin of the adventive *G. kimorum* populations in Pacific Northwest is unclear, as is whether they originated from multiple sources. A recent genomic analysis showed a high similarity of the currently released Tokyo strain and individuals collected from the British Colombian population and *Wolbachia* sequences were found in the genomic sequencing of different populations (Hopper et al. 2024). Our results provide evidence that the currently released and adventive populations are not only genetically identical but also reproductive compatible. In our crossbreedings between the released and the adventive populations, combinations of both sexes were prepared, and for both combinations ('rel' female × 'adv' male as well as 'adv' female × 'rel' male) proof of successful mating could be obtained, suggesting reproductive compatibility. Our results also ruled out potential cytoplasmic incompatibility between populations caused by a symbiotic Wolbachia. A previous test with the Tokyo *G. kimorum* females also revealed that unmated females produced male offspring only, i.e., ruling out the possibility of thelytoky due to *Wolbachia* infection in this species (Hopper et al. 2024). We can therefore expect interactions between released and adventive *G. kimorum* in North America might occur. Future studies should investigate the origin of adventive *G. kimorum* populations in the Pacific Northwest and the potential effects of admixture on their fitness and effectiveness as biological control agents. By carefully considering the genetic diversity and ecological characteristics of different *G. kimorum* populations, we can optimize the introduction of biological control agents and improve their long-term success.

This study has practical implications for the classical biological control program of *D. suzukii*. Since the laboratory colony descends from only a small subset of the adventive population, our results cannot eliminate the possibility of additional species within the *G. brasiliensis* species group being present in the adventive range, which might have less or no compatibility with the released material. However, aligning with Beers et al. (2022), this study is further evidence of adventive *G. kimorum*, the specific agent with government approval for release in Italy and the USA. Having successfully established in North America, the adventive *G. kimorum* population will likely be well-adapted to climate conditions in the adventive range. This creates an opportunity for re-distribution from the adventive range to other locations within the administrative region (depending on the legislation of the relevant state, province, or country), as has been considered or realized for other biological control agents (Braman et al. 2021; Bergh et al. 2023).

Conclusion

Our results settle potential concerns about mating incompatibility and pave the way for re-distribution in the USA of the adventive *G. kimorum* population and imported *G. kimorum* from Tokyo. Overall, this study contributes to a better understanding of the reproductive status of two *G. kimorum* populations being used for classical biological control and improves predictions of the outcome of field releases for classical biological control against *D. suzukii* in North America. Still there is the possibility of viability or phenotypic effects resulting from the admixture of the two populations that has yet to be determined.

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